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Peptide specificity, HLA class II restriction, and T-cell subsets of the T-cell clones specific to either Cry j 1 or Cry j 2, the major allergens of Japanese cedar (Cryptomeria japonica) pollen.

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BACKGROUND: Cry j 1 and Cry j 2 are thought to be the major allergens of Japanese cedar pollen. HLA class II types capable of presenting T-cell epitopes in both allergens and their role in induction of T-cell subsets are not well known. METHODS: CD4+ T (Th)-cell clones (TCCs) specific to either Cry j 1 or Cry j 2 were generated. HLA class II restrictions were determined by their reactivity to the T-cell epitope in the presence of antigen presenting cells sharing matched types. Interleukin (IL)-2, interferon-gamma, IL-4, and IL-5 contents in the supernatants of TCCs were estimated using enzyme immunoassay. RESULTS: Peripheral blood mononuclear cells (PBMC) from patients induced proliferation with 100 microgram/ml Cry j 1 or 3-10 microgram/ml rCry j 2 stimulation. T-cell epitopes in Cry j 1 were presented to Th cells by the gene products of DRA1*01/DRB1*0901, DRA1*01/DRB5*0101, DQA1* 0102/DQB1*0602, and DPA1*01/DPB1*0501; those in Cry j 2 were restricted by DRA1*01/DRB1*0901, DRA1* 01/DRB1*1501, DRA1*01/DRB4*01, DRA1*01/DRB5* 0101, DQA1*0102/DQB1*0602, DPA1*01/DPB1*0201, and DPA1*01 and *0202/DPB1*0501. Type 2-like cells were preferentially induced in Cry j 1 stimulation, while an almost equal number of type 2- and type 1-like cells was induced in rCry j 2. CONCLUSIONS: No clear correlation existed between peptide specificity, HLA class II restriction and induction of Th-cell subsets, suggesting that the requirement of different dose of Cry j 1 or Cry j 2 to induce proliferation in PBMC may lead to distinguishable difference in induction of Th subsets between TCCs specific to Cry j 1 and Cry j 2.

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Peptide Specificity, HLA Class II Restriction, and T-Cell Subsets of the T-Cell Clones Specific to Either Cry j 1 or Cry j 2, the Major Allergens of Japanese Cedar *(Cryptomeria japonica)* Pollen

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Key Words
Cry j 1 · Cry j 2 · T-cell clone · HLA class II restriction · T-cell epitopes · Th1/Th2

Abstract
Background: Cry j 1 and Cry j 2 are thought to be the major allergens of Japanese cedar pollen. HLA class II types capable of presenting T-cell epitopes in both allergens and their role in induction of T-cell subsets are not well known. ***Methods:*** CD4+ T (Th)-cell clones (TCCs) specific to either Cry j 1 or Cry j 2 were generated. HLA class II restrictions were determined by their reactivity to the T-cell epitope in the presence of antigen presenting cells sharing matched types. Interleukin (IL)-2, interferon-γ, IL-4, and IL-5 contents in the supernatants of TCCs were estimated using enzyme immunoassay. ***Results:*** Peripheral blood mononuclear cells (PBMC) from patients induced proliferation with 100 μg/ml Cry j 1 or 3–10 μg/ml rCry j 2 stimulation. T-cell epitopes in Cry j 1 were presented to Th cells by the gene products of DRA1*01/DRB1*0901, DRA1*01/DRB5*0101, DQA1*0102/DQB1*0602, and DPA1*01/DPB1*0501; those in Cry j 2 were restricted by DRA1*01/DRB1*0901, DRA1*01/DRB1*1501, DRA1*01/DRB4*01, DRA1*01/DRB5*0101, DQA1*0102/DQB1*0602, DPA1*01/DPB1*0201, and DPA1*01 and *0202/DPB1*0501. Type 2-like cells were preferentially induced in Cry j 1 stimulation, while an almost equal number of type 2- and type 1-like cells was induced in rCry j 2. ***Conclusions:*** No clear correlation existed between peptide specificity, HLA class II restriction and induction of Th-cell subsets, suggesting that the requirement of different dose of Cry j 1 or Cry j 2 to induce proliferation in PBMC may lead to distinguishable difference in induction of Th subsets between TCCs specific to Cry j 1 and Cry j 2.

Introduction

Japanese cedar *(Cryptomeria japonica)* pollen is a potent seasonal aeroallergen in Japan affecting more than 10% of the population with cedar pollinosis [1]. Two major allergenic proteins, Cry j 1 and Cry j 2, have been isolated [1, 2] and both are thought to be important in the immunopathology of Japanese cedar pollinosis, since IgE specific to Cry j 1 was detected in up to 95% of the patients, while that to Cry j 2 in about 70% [2–5]. Determination of primary structures of the Cry j 1 [6] and Cry j 2 [3, 7] was followed by the identification of multiple T-cell epitopes in both sequences [8–10].

Human Th cells stimulated by a particular antigen can be classified as one of subsets of cell, type 1-like Th (Th1-

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like), type 2-like Th (Th2-like), and intermediate type 0-like Th (Th0-like) cells, on the basis of their interleukin(IL)-4 and interferon(IFN)-γ secretion profile. Following allergen exposure, Th cells from allergic patients preferentially differentiate into Th2- rather than Th1-like cells [11–14]. Little information about induction of the subsets of Th cells in response to Cry j 1 and Cry j 2 stimulations is now available.

HLA class II molecules are classified into three major isotypes, DR, DQ, and DP. The molecules are postulated to be directly or indirectly relevant to the pathophysiology of allergic diseases due to their function as antigen presenting molecule and the associations of their particular types with specific IgE responsiveness to various sources of allergen [15–17]. Actually, several types of HLA class II DR, DQ, and DP molecules were shown to function as restriction molecule for the presentation of T-cell epitopes in Der p 1 [18], Der p 2 [19], Fel d 1 [20], Asp f 1 [21], Der f 2 [22] and so on. Recently, the gene products of DRA1*01/DRB3*0301 and DPA1*0202/DPB1*0501 were shown to be capable of presenting Cry j 1 p335-346 [23] and p214-222 [24], respectively. Further studies of determination of the types of HLA class II molecules capable of presenting T-cell epitopes in Cry j 1 and Cry j 2 are necessary to elucidate whether there is any overall association between Japanese cedar pollinosis and HLA class II types.

To simplify HLA class II restriction, we selected 4 allergic patients having HLA class II-types, one is DRB1*1501 and another is one of three types, DRB1*0405, DRB1*0802, and DRB1*0901, frequently found in the Japanese population [25] among 18 allergic patients [10]. Peptide specificity, HLA class II restriction, and subsets of the T-cell clones (TCCs) specific to either Cry j 1 or Cry j 2 generated from the patients are determined. Based on these observations, we show whether peptide specificity and HLA class II restriction is associated with the preferential induction of subsets of TCCs specific to either Cry j 1 or Cry j 2 and discuss the possible role of HLA class II molecules in immunopathology of Japanese cedar pollinosis.

Materials and Methods

Blood Donors and HLA Class II Typing

Blood donors (4 males aged 31–44) were allergic patients suffering from Japanese cedar pollinosis, a diagnosis made on the bases of their case histories and the presence of allergen-specific IgE as measured by Ala-STAT (Diagnostic Products, Los Angeles, Calif., USA). All patients and healthy individuals (2 males aged 31 and 43) gave informed consent. Genotypes of HLA class II loci (DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPA1, and DPB1) of the patients were determined by polymerase chain reaction/sequence-specific oligonucleotide probe analysis [26] using DNA prepared from peripheral blood mononuclear cells (PBMC).

Allergens

Cry j 1 was purified by a well-established procedure [1] from *C. japonica* pollen collected in Atami, Shizuoka Prefecture, Japan.

The existence of two mature forms of Cry j 2, probably due to complete and incomplete processing of the protein, has been reported [3, 7]. One is composed of 379 amino acid residues whose sequence corresponds to the translation product of the mRNA encoding Cry j 2 at positions from 55 to 434 and another is 388 residues at positions from 46 to 434, respectively. Expression of rCry j 2 (from Ala at position 55 to a *C*-terminus) in *Escherichia coli* was performed according to the procedure described elsewhere [10]. Purified rCry j 2 was dissolved in 8 *M* urea/phosphate-buffered saline (PBS) and then adjusted to 5–10 mg/ml. The protein concentration was determined using a BCA Kit (Pierce, Rockford, Ill., USA). The purified protein was detected as one band (>90% purity) on a SDS-PAGE gel after staining with Coomassie Brilliant Blue R-250.

Synthesis of Peptides

Two panels of 69 and 74 overlapping peptides, each 15 amino acids long with 10 amino acid overlap, covered with entire sequences of the Cry j 1 (PCCI2-2) [6] and Cry j 2 (PCII-1) [3], respectively, were synthesized according to a procedure described previously [10] using a solid phase peptide synthesizer, PSSM-8 (Shimadzu, Kyoto, Japan), which employs the F-moc strategy. Each peptide was purified by a reverse-phase high-performance liquid chromatography (Waters, Milford, Mass., USA) using a Protein C4 column (Vydac, Hesperia, Calif., USA). Purity of the peptides was estimated to >90%, as judged by the height of a main peak by mass spectrometry (Kompact Maldi I, Shimadzu). After lyophilization, each peptide was dissolved in 8 *M* urea/PBS to a concentration of 2 m*M*. 300-fold dilution of 8 *M* urea/PBS containing each peptide and rCry j 2 with culture media did not show any cytotoxic effect on the cells used for the culture.

Antigen Presenting Cells (APC)

PBMC were isolated from heparinized blood by density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). Epstein-Barr virus-transformed B (EBV-B) cells were established by cultivation of PBMC after in vitro infection of EBV, which was obtained from a culture of the marmoset cell line B95-8 (kindly provided by Dr. K. Yamamoto, Tokyo Medical and Dental University, Tokyo, Japan) in the presence of 1 μg/ml Cyclosporin A (Novartis, Basel, Switzerland). EBV-B cells (EA, T7526, BM9, and BOB) were kindly provided by Dr. K. Hirayama (Saitama Medical School, Saitama, Japan). EBV-B cells were cultured in RPMI-1640 (Nissui Pharmaceutical, Osaka, Japan) supplemented with 10–12% FCS (Life Technologies, Grand Island, NY, USA). L cells expressing a set of a DR-α chain (DRA1*0101) and a polymorphic DR-β chain (DRB1*1501, DRB1*0405, DRB1*0901, DRB4*0101 and DRB5*0101), were kindly provided by Dr. Y. Nishimura (Kumamoto University, Kumamoto, Japan). The cells were cultured in Dulbecco's MEM (Nissui Pharmaceutical) supplemented with 10% FCS. EBV-B cells and L cells were treated with 50 μg/ml mitomycin C (MMC) (Kyowa Hakko, Tokyo, Japan) for 30 min followed by four washings with RPMI-1640 after which they were used as APC.

Generation of Cry j 1- and Cry j 2-Specific T-Cell Lines (TCLs) and TCC

PBMC (4×10^6) were cultured with 50 μg/ml Cry j 1 or 10 μg/ml rCry j 2 in 2 ml of medium in one well of a 24-well plate for 8 days. The medium used was RPMI-1640 supplemented with 15% human AB serum and antibiotics (streptomycin and penicillin (Life Technologies)) (complete medium). T-cell blasts obtained were utilized for the generation of allergen-specific TCLs and TCCs as described below.

The T-cell blasts were washed twice with PBS and were cultured in complete medium in the presence of 20 U/ml rIL-2 (Boehringer, Manheim, Germany). The culture was continued for an additional 12–14 days. The medium was changed every day during culture. An aliquot of TCLs was used for proliferation experiments in the absence of rIL-2 and the remainder was stored in liquid N_2. TCLs showing stimulation index (SI) >3 was used for the determination of T-cell epitopes [10].

An aliquot of the T-cell blasts was transferred to a 15-ml tube and then diluted 100-fold with complete medium. 5 ml of the cell suspension was transferred to a 10-cm diameter culture dish. One T-cell blast was separated using the tip of a micropipet under the microscope and was transferred to one well of a 96-well round-bottomed plate to which 2×10^5 MMC-treated autologous EBV-B cells in 0.2 ml complete medium containing 25 μg/ml Cry j 1 or 5 μg/ml rCry j 2 and 20 U/ml rIL-2 had been previously placed. Finally, 258 and 266 T-cell blasts from Cry j 1-stimulated PBMC of PB and PJ, respectively, and 229, 154, and 135 T cell blasts from rCry j 2-stimulated PBMC of PB, PC, and PR, respectively, were transferred to the well. After stimulation of the cells twice with Cry j 1 or rCry j 2 on the same condition at 7-day intervals, growing cells appeared in about one half of the wells. The cells were transferred to a well of a 24-well culture plate and further stimulated once or twice under the same conditions. An aliquot of the cells was used for proliferation experiment in the absence of rIL-2. TCCs showing SI>5 were selected and were stored in liquid N_2 prior to use. Cloning efficiencies for TCCs specific to Cry j 1 and rCry j 2 were calculated from 11 to 20% and from 9 to 18%, respectively, depending on the appearance of T-cell blasts. About one third of TCCs specific to the allergens was well grown and was available for following experiments. The phenotype of TCCs was determined by flow cytometry using a FACStar (Becton-Dickinson, Mountain View, Calif., USA) after staining them with FITC-conjugated anti-CD3 (Leu-4), anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-Tαβ (TCRα/β), and anti-Tγδ (TCR-γ/δ-1) monoclonal antibodies (mAb) (all from Becton-Dickinson).

T-cell Proliferative Response

PBMC (2×10^5) were cultured in 0.2 ml complete medium at various concentrations of Cry j 1 or rCry j 2 in 96-well round-bottomed plates for 7 days. Proliferation of TCLs and TCCs was assayed by co-culturing the cells (2×10^4) with MMC-treated autologous EBV-B cells (5×10^4), allogeneic EBV-B cells sharing an HLA class II haplotype, and L cells (4×10^4) as APC in 0.2 ml of complete medium in 96-well flat-bottomed culture plates. Synthetic overlapping peptides were added to each well to a final concentration of 1 μ*M*, corresponding to a 38 μg/ml Cry j 1 dose, or 0.5 μ*M*, corresponding to a 21 μg/ml rCry j 2 dose, and the cells were incubated for 72 h. To determine the restriction molecule, one of anti-DR (L243), anti-DQ (Leu-10), and anti-DP (B7/21) mAbs (Becton-Dickinson) was added to each well to a final concentration of 0.6 μg/ml. For the last 16 h incubation, 0.5 μCi of [^{3}H]thymidine (NEN, Boston, Mass., USA) was added to each culture. After harvest of the cells, incorporation of [^{3}H]thymidine was measured using a BETAmatic II Liquid Scintillation Counter (Kontron, Basel, Switzerland). All cultures were performed in triplicate.

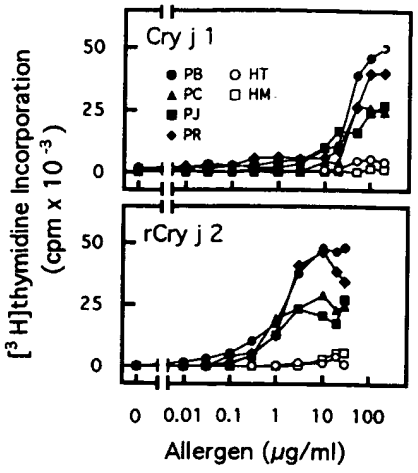

Fig. 1. Proliferative response of PBMC under stimulation with Cry j 1 and rCry j 2. PBMC from 4 allergic patients (PB, PC, PJ, and PR) and 2 healthy individuals (HT and HM) were cultured with 0–200 μg/ml Cry j 1 or 0–30 μg/ml rCry j 2 for 7 days. For the last 16 h incubation, [^{3}H]thymidine was added to each well. After harvest of the cells, incorporation of [^{3}H]thymidine was measured. The results shown are the mean cpm of triplicate cultures (SEM < 10%).

Measurement of Lymphokines

TCCs (5×10^5) were co-cultured with 1×10^6 MMC-treated autologous EBV-B cells in 1 ml of RPMI-1640 supplemented with 5% human AB serum in the presence of 1 μ*M* of particular peptide from Cry j 1 and 0.5 μ*M* from Cry j 2 for 24 h. After gentle centrifugation, culture supernatant obtained was stored at –20°C prior to use. The amounts of IL-2 (≥31 pg/ml detection; R&D Systems, Minneapolis, Minn., USA), IL-5 (≥16 pg/ml; R&D Systems), IL-4 (≥31 pg/ml; Endogen, Cambridge, Mass., USA), and IFN-γ (≥31 pg/ml; Otsuka Pharmaceuticals, Osaka, Japan) in the supernatant were measured using a sandwich-enzyme immunoassay technique. In such cases where the amounts of lymphokines appeared over the range of detection, the supernatant was 10-fold diluted with complete medium and the contents were further measured. Cultures were set up in duplicate and the average value was indicated.

Results

Proliferative Response of PBMC to Cry j 1 and rCry j 2 Stimulation

Cry j 1 or rCry j 2 stimulation induced proliferative responses in PBMC from 4 allergic patients in a dose-dependent manner (fig. 1). The same stimulation did not induce

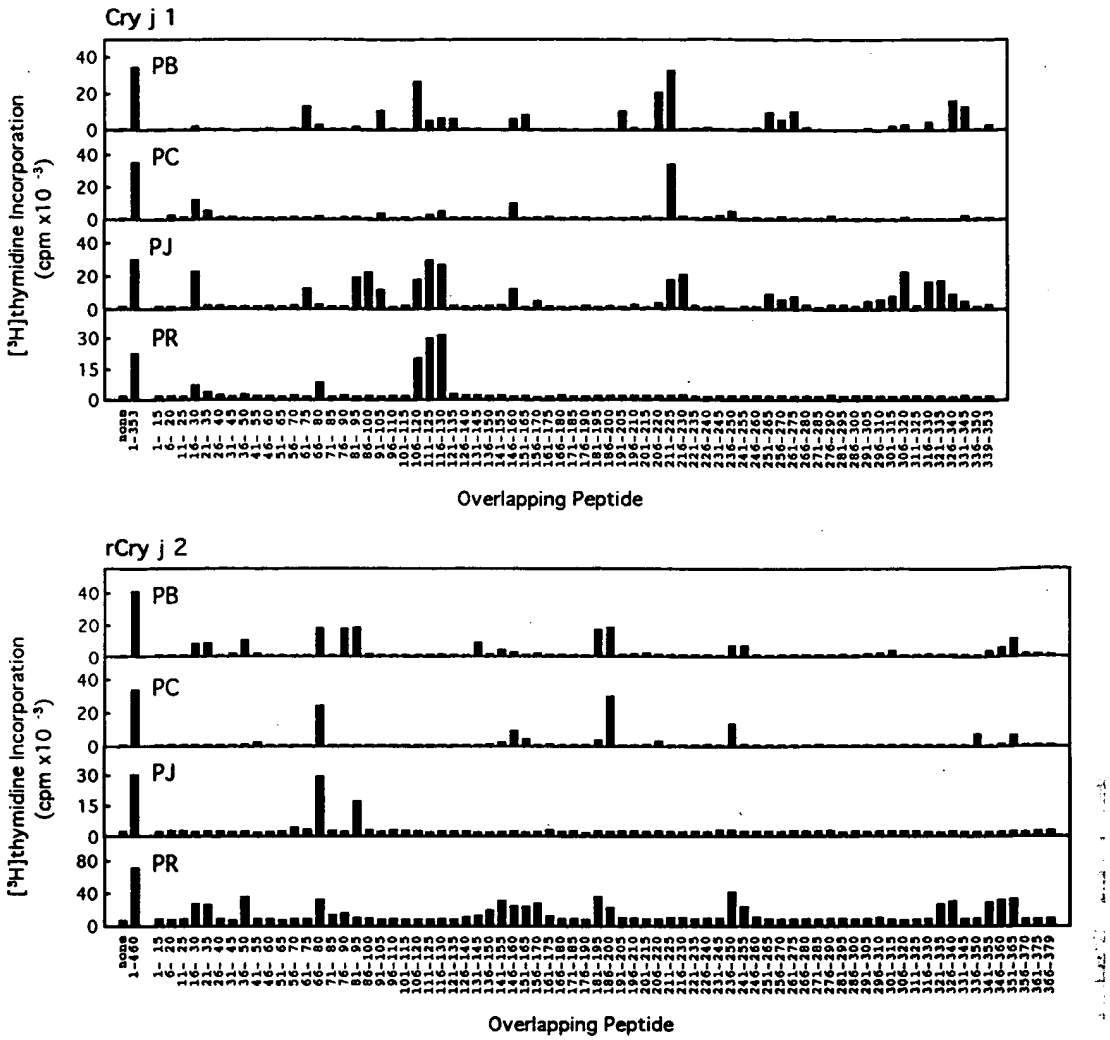

Fig. 2. Proliferative response of TCLs specific to either Cry j 1 or rCry j 2 by stimulation with a panel of overlapping peptides covered with Cry j 1 or Cry j 2 sequences. TCLs were co-cultured with MMC-treated autologous EBV-B cells for 3 days in the presence of 25 μg/ml Cry j 1, 5 μg/ml rCry j 2, 1 μ*M* overlapping peptide from the Cry j 1 sequence and 0.5 μ*M* from Cry j 2. For the last 16 h incubation, [3H]thymidine was added to each well. After harvest of the cells, incorporation of [3H]thymidine was measured. The mean value of triplicate cultures is shown (SEM < 10%).

proliferative response in PBMC from 2 healthy individuals. The PBMC response appeared to reach a plateau at a concentration of 100 μg/ml Cry j 1 and 3–10 μg/ml rCry j 2.

Generation of TCLs and TCCs Specific to Either Cry j 1 or rCry j 2

Cry j 1- and rCry j 2-specific TCLs were generated from PBMC of the 4 allergic patients, since the PBMC occasionally induced very low proliferative response, to an extent of SI<3, to the stimulation with 2 μ*M* overlapping peptides (data not shown) [10]. Identification of T-cell epitopes in the Cry j 1 was examined by the reactivity of TCLs to the stimulation with a panel of overlapping peptides from Cry j 1 sequence in the presence of autologous EBV-B cells as APC. The same experiment was performed in TCLs specific to rCry j 2 to the peptides from Cry j 2 sequence. As shown in figure 2, the TCLs could recognize several limited regions in Cry j 1 and Cry j 2 sequences as T-cell epitopes.

TCCs specific to Cry j 1 were generated from PBMC of 2 patients (PB and PJ); those specific to rCry j 2 were generated from three (PB, PC, and PR). In total, 29 TCCs specific to Cry j 1 and 30 TCCs reactive to rCry j 2 were used for a series of following experiments. The phenotype of all TCCs determined by flow cytometry was CD3+, CD4+, and CD8–. TCR expressed on the cells was αβ+ and γδ- (data not shown).

To set up experimental conditions, several TCCs specific to Cry j 1 or rCry j 2 were selected and their reactivity to a panel of overlapping peptides examined. A representative of the results was shown in figure 3a. Two TCCs, PB8-4 specific to Cry j 1 p251-265 and PB4-22 to Cry j 2 p66-80, were reactive to the peptides in a dose-dependent manner. After that, TCCs were stimulated with 1 μ*M* overlapping peptide from Cry j 1 sequence or 0.5 μ*M* from Cry j 2 throughout experiments. Identification of T-cell epitopes recognized by individual TCCs was examined by their reactivity to a panel of overlapping peptides from the Cry j 1 or Cry j 2 sequence in the presence of autologous EBV-B cells. Each TCC generated was reactive to one of the T-cell epitopes identified in TCLs (fig. 2) (data not shown).

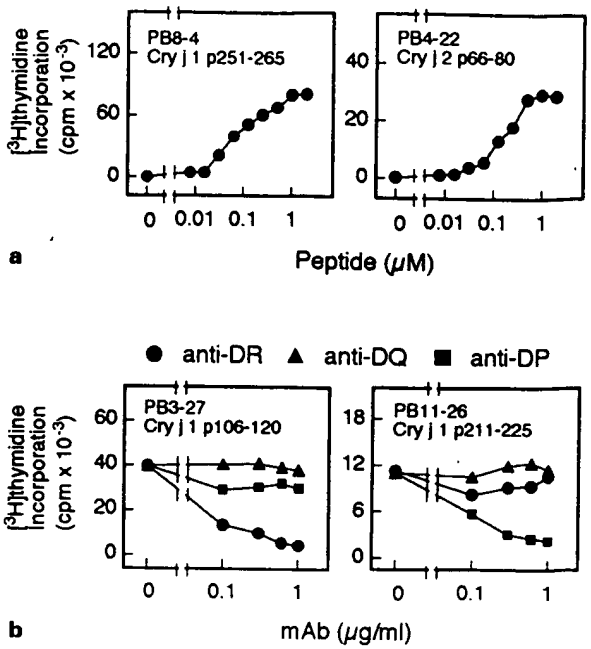

Fig. 3. Proliferation of Cry j 1- and Cry j 2-specific TCCs stimulated by a particular T-cell epitope (**a**) and inhibition of proliferation in Cry j 1-specific TCCs in the presence of anti-DR, DQ, or DP mAb (**b**). Two TCCs, PB8-4 and PB4-20, were cultured for 3 days in the presence of 0–2 μ*M* denoted peptide. Two TCCs, PB3-27 and PB11-26, were cultured for 3 days in the presence of a denoted peptide at a final concentration of 1 μ*M* and one of anti-DR, -DQ, and -DP mAbs. For the last 16 h incubation, [^{3}H]thymidine was added to each well. After harvest of the cells, incorporation of [^{3}H]thymidine was measured. Cultures are set up in triplicate and the mean value is indicated (SEM < 10%).

HLA Class II-Restriction of TCCs

In a preliminary experiment, proliferation inhibition assay was examined using PB3-27 and PB11-26 specific to Cry j 1 in the presence of one of anti-DR, -DQ and -DP framework mAbs (fig. 3b). Proliferation of two TCCs was inhibited by the addition of one of mAbs in a dose-dependent manner. Presence of 0.6 μg/ml mAb in our culture condition was enough to determine HLA class II restriction of TCCs. Determination of the restriction molecule for each TCC was examined. As shown in table 1, DR, DQ, and DP molecules could function as restriction molecule for the presentation of T-cell epitopes from Cry j 1 and Cry j 2. One peptide, Cry j 1 p191-205, was restricted by DR as well as DQ molecule.

HLA class II genotypes of 4 allergic patients and EBV-B cells utilized as APC are listed in table 2. To identify individual restriction molecule, TCCs restricted by DR were co-cultured with L cells expressing a set of a nonpolymorphic DR-α chain (DRA1*0101) and one of polymorphic DR-β chain (DRB1*0405, DRB1*0901, DRB1*1501, DRB4*0101, and DRB5*0101) and also with allogeneic EBV-B cells, EB-PK (DRB1*0802/0803). To determine DQ and DP restriction, EBV-B cells sharing an HLA class II haplotype selected and used as APC. Proliferative responses of all TCCs to the stimulation with individual peptides containing T-cell epitopes were examined. A part of the proliferative responses of Cry j 1- and Cry j 2-specific TCCs restricted by several types of the gene products of DR, DQ, and DP loci derived from a patient PB is shown in figure 4. Each restriction molecule for all TCCs is summarized in table 3. The gene products of DRA1*01/DRB1*0901, DRA1*01/DRB5*0101, DQA1*0102/DQB1*0602, DPA1*01/DPB1*0501 could present one of T cell epitopes in the Cry j 1 sequence to TCCs. PB11-23 and PB13-18, restricted by a gene product of DPA1*01/DPB1*0501, induced a weak proliferation to the stimulation with each peptide presented by EB-PQ (DPA1*0202/DPB1*0501). No peptides were presented by the gene products of DRA1*01/DRB1*0802, DRA1*01/DRB1*1501, DRA1*01/DRB4*01, DQA1*0401/DPB1*0402, DQA1*0301/DQB1*0303, DPA1*01/DPB1*0201, and DPA1*ND/DPB1*0402. The gene products of DRA1*01/DRB1*0901, DRA1*01/DRB1*1501, DRA1*01/DRB4*01, DRA1*01/DRB5*0101, DQA1*0102/DQB1*0602, DPB1*01/DPB1*0201,

Table 1. Inhibition of proliferation of TCCs specific to either Cry j 1 or rCry j 2 by anti-HLA class II mAbs[a]

TCC[b] specific to	Proliferation (cpm×10^{-3})				Restriction
	none	α-DR	α-DQ	α-DP	
Cry j 1					
PJ4-6	4.9	4.2	0.2	3.4	DQ
PB10-24	22.0	33.9	14.7	3.4	DP
PJ1-27	3.0	2.0	0.2	2.7	DQ
PB3-27	70.1	1.4	61.3	61.6	DR
PJ5-6	78.3	56.7	2.5	64.0	DQ
PB11-21	16.8	0.1	12.7	13.0	DR
PB1-8	27.5	33.0	1.4	19.5	DQ
PB9-34	19.5	0.1	18.3	16.2	DR
PB11-23	20.6	29.3	23.0	0.7	DP
PB8-4	72.0	67.1	8.2	54.6	DQ
PJ4-20	6.6	12.9	0.2	8.4	DQ
rCry j 2					
PB11-40	2.8	0.2	2.3	1.5	DR
PR5-40	62.9	5.3	57.7	38.0	DR
PB4-22	15.0	0.2	15.5	16.8	DR
PB13-18	34.7	37.6	27.9	0.5	DP
PR1-20	12.5	0.4	9.2	5.3	DR
PB14-5	25.1	20.8	18.8	2.0	DP
PB14-34	16.5	0.0	10.6	14.2	DR
PB5-3	50.2	0.0	41.2	39.9	DR
PR2-34	45.4	1.4	37.7	40.4	DR
PR3-30	6.7	3.3	0.9	8.1	DQ
PC1-13	63.6	84.6	36.6	0.4	DP
PR5-12	47.1	42.4	0.2	32.7	DQ
PB3-38	27.7	15.1	0.5	20.8	DQ

[a] TCCs were co-cultured with autologous EBV-B cells for 3 days in the presence of a T-cell epitope containing peptide and one of three mAbs.

[b] All TCCs generated were examined. An example of the TCC is shown, in case that proliferative response of two or more TCCs reactive to the same T-cell epitope was inhibited by the addition of the same mAb.

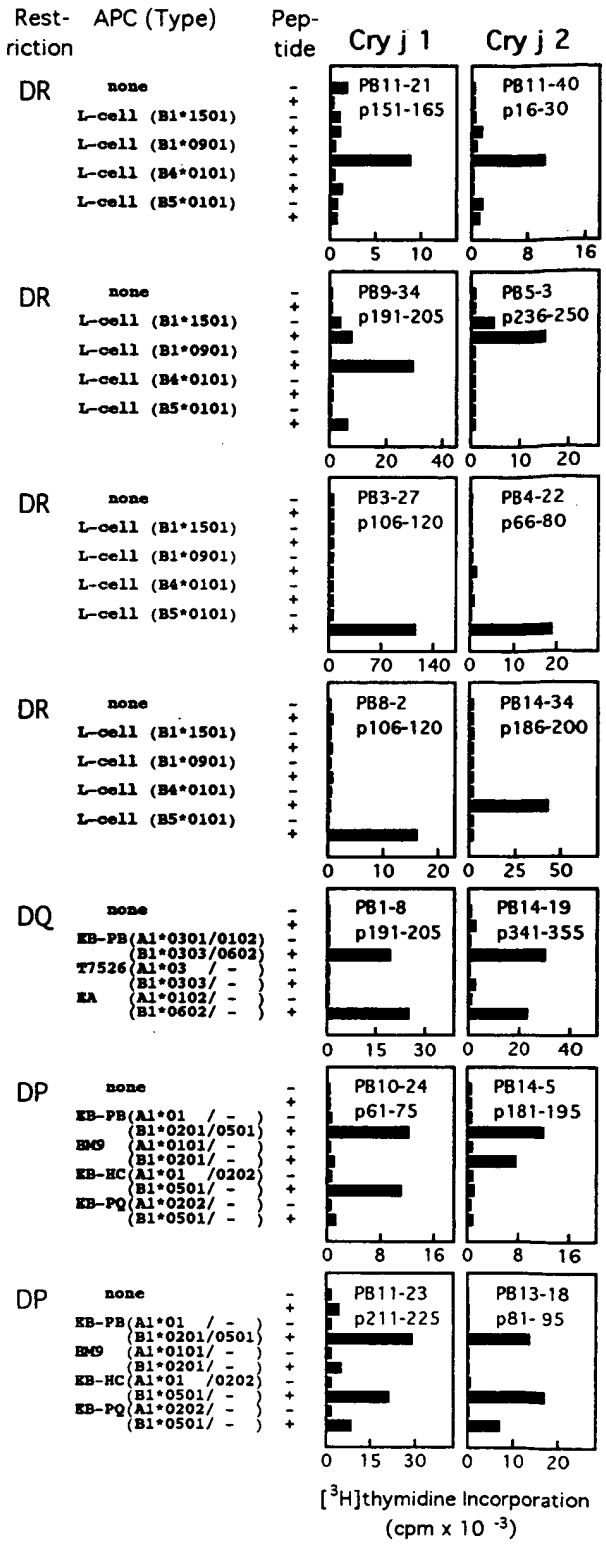

Fig. 4. HLA class II restriction of TCCs specific to either Cry j 1 or rCry j 2. TCCs generated from a patient PB were co-cultured with the denoted L-cell transfectants expressing an individual DR type or EBV-B cells sharing an HLA class II haplotype for 3 days in the presence of 1 μ*M* peptide from the Cry j 1 sequence or 0.5 μ*M* peptide from Cry j 2 whose sequence is denoted in the column. For the last 16 h incubation, [^{3}H]thymidine was added to each well. After harvest of the cells, incorporation of [^{3}H]thymidine was measured. A part of the TCCs analyzed is shown. Cultures are set up in triplicate and the mean value is indicated (SEM < 10%).

Table 2. HLA class II genotypes of the allergic patients and EBV-B cells as APC

Cells		DR				DQ		DP	
source	name	DRB1*	DRB3*	DRB4*	DRB5*	DQA1*	DQB1*	DPA1*	DPB1*
Patient	PB	1501/0901		01	0101	0102/0301	0602/0303	01 /–	0501/0201
	PC	1501/0405		01	0101	0102/0301	0602/0401	0202/–	0501/0201
	PJ	1501/0802			0101	0102/0401	0602/0402	ND[a]/ND	0501/0402
	PR	1501/0901		01	0101	0102/0301	0602/0303	01 /0202	0201/–
EBV-B cell	EA[b]	1501/–			0101/–	0102/–	0602/–	01 /–	0401/–
	T7526[b]	0901/–		01/–		03 /–	0303/–	0401/–	1301/–
	BM9[b]	0801/–				0401/–	0402/–	01 /–	0201/–
	BOB[b]	1104/–	02/–			0501/–	0301/–	01 /–	0402/–
	EB-HC[c]	0901/–		01/–		0301/–	0303/–	01 /0202	0501/–
	EB-PK[c]	0802/0803				0401/0103	0402/0601	0201/0202	0202/0501
	EB-PQ[c]	0405/0803		01		0301/0103	0401/0601	0202/–	0501/–

[a] Not determined.
[b] Distributed by 9th International Histocompatibility Workshop.
[c] Established in our laboratory.

and DPA1*01 and *0202/DPB1*0501 could present one of T-cell epitopes in the Cry j 2 sequence to TCCs, while no peptides were presented by the gene products of DRA1*01/DRB1*0405, DRA1*01/DRB1*0802, DQA1*0301/DQB1*0401, DQA1*0401/DQB1*0402, DQA1*0301/DQB1*0303 and DPA1*ND/DPB1*0402.

Lymphokine Secretion by TCCs

The amounts of IL-2, IFN-γ, IL-4, and IL-5 secreted by TCCs were measured by enzyme immunosorbent assay. The results of peptide specificity, HLA class II restriction, lymphokine secretion, and subsets of the TCCs specific to either Cry j 1 or Cry j 2 are shown in tables 4 and 5. Of the 29 TCCs specific to Cry j 1, 1 (3%) was characterized as Th1-, 10 (34%) as Th2-, and 16 (55%) as Th0-like cells, on the basis of IL-4/IFN-γ secretion profile. Cry j 1 stimulation preferentially induced Th2- rather than Th1-like cells. Of the 30 TCCs reactive to rCry j 2, 7 (23%) were classified into Th1-, 8 (27%) into Th2-, and 8 (27%) into Th0-like cells. An almost equal number of Th1- and Th2-like cells was induced.

IL-5 was secreted by all TCCs and the average level of IL-5 secreted was highest among the lymphokines measured.

We examined whether a combination of peptide specificity and HLA class II restriction could control preferential induction of Th subsets. Among TCCs reactive to Cry j 1, Th2-like cells randomly appeared irrespective of the peptide specificity and DR, DQ, and DP restriction. As for TCCs specific to Cry j 2, both Th1- and Th2-like cells were induced under a combination of a peptide and a certain type of HLA class II molecule. A combination of Cry j 1 p61-75 and DPA1*01/DPB1*0501 tended to induce Th2-like cells in TCCs from PB, while a combination of Cry j 2 p36-50 and DPA1*01/DPB1*0501 could induce Th1- and Th2-like cells in TCCs from PB. A set of Cry j 2 p341-355 and DQA1*0102/DQB1*0602 resulted in induction of the Th1-like cells in TCCs from PB and the Th2-like cells from PC, suggesting that induction of Th1- and Th2-like cells is different among individuals. Taken together, differentiation of the Th cells toward Th1- or Th2-like cells appeared to be independent of a specific combination of a peptide and a type of HLA class II molecule.

Discussion

Cumulative data of the identification of T-cell epitopes in clinically important allergenic proteins [18–22, 27, 28] revealed that multiple regions in sequences of the proteins are recognized as T-cell epitopes. Recently, existence of multiple T-cell epitopes in Cry j 1 [9, 10] and Cry j 2 sequences [8, 10] was reported. The results of identification of T-cell epitopes in Cry j 1 and Cry j 2 sequences, based on 4 allergic patients studied, are consistent with previous reports and are reproducible. These observations propose that individual T-cell epitopes in both sequences are postulated to be presented to Th cells by several types of HLA class II

Table 3. Peptide specificity and HLA class II restriction of all TCCs

Epitope position	TCC					Restriction molecule derived from
Cry j 1						
16–30	PB8-1					DQA1*0102/DQB1*0602
61–75	PB9-37	PB10-24				DPA1*01/DPB1*0501
106–120	PB3-27	PB8-2	PB8-3	PB9-39	PB10-18	DRA1*01/DRB5*0101
151–165	PB6-37	PB11-21	PB11-24			DRA1*01/DRB1*0901
191–205	PB1-8					DQA1*0102/DQB1*0602
191–205	PB9-34					DRA1*01/DRB1*0901
211–225	PB2-14	PB4-20	PB7-2	PB8-32	PB8-34	DPA1*01/DPB1*0501
	PB10-4	PB11-23	PB11-26			
251–265	PB8-4					DQA1*0102/DQB1*0602
16–30	PJ4-6					DQA1*0102/DQB1*0602
91–105	PJ1-27					DQA1*0102/DQB1*0602
106–120	PJ4-29	PJ7-9				DRA1*01/DRB5*0101
146–160	PJ5-6	PJ5-9				DQA1*0102/DQB1*0602
326–340	PJ4-20					DQA1*0102/DQB1*0602
Cry j 2						
16–30	PB5-29	PB11-40	PB14-4			DRA1*01/DRB1*0901
36–50	PB12-33					DRA1*01/DRB1*0901
66–80	PB3-32	PB4-21	PB4-22			DRA1*01/DRB5*0101
81–95	PB11-32	PB13-18				DPA1*01/DPB1*0501
181–195	PB14-5	PB14-13				DPA1*01/DPB1*0201
186–200	PB14-34					DRA1*01/DRB4*01
236–250	PB5-3					DRA1*01/DRB1*1501
341–355	PB12-8	PB14-19				DQA1*0102/DQB1*0602
346–360	PB13-38					DQA1*0102/DQB1*0602
66–80	PC1-8	PC3-21				DRA1*01/DRB5*0101
186–200	PC3-40					DRA1*01/DRB4*01
336–350	PC1-13					DPA1*0202/DPB1*0501
36–50	PR2-25	PR5-40				DRA1*01/DRB1*0901
66–80	PR4-20					DRA1*01/DRB5*0101
151–165	PR1-20	PR4-39				DRA1*01/DRB1*0901
321–335	PR2-34					DRA1*01/DRB1*0901
326–340	PR3-30	PR5-18				DQA1*0102/DQB1*0602
341–355	PR2-31	PR5-12				DQA1*0102/DQB1*0602

molecules. Indeed, various types of DR, DQ and DP molecules were shown to present T-cell epitopes from Der p 1 [18], Der p 2 [19, 27], Fel d 1 [20], and Asp f 1 [21] and so on. As for Cry j 1, the gene products of DRA*01/DRB3*0301 and DPA1*0202/DPB1*0501 could present Cry j 1 p335-346 [23] and p214-222 [24], respectively. In the present study, we clearly demonstrated that several types of the gene products of HLA class II, DR, DQ, and DP loci could present T-cell epitopes in Cry j 1 and Cry j 2 sequences (tables 4, 5). Antigen frequencies of DRB1*0901, DRB1*1501, DRB4*01, DRB5*0101, DQB1*0602, DPB1*0201, and DPB1*0501 in the Japanese population are 0.24, 0.12, 0.50, 0.12, 0.12, 0.49, and 0.65, respectively [25]. HLA class II molecules whose types are frequently found in the Japanese population could present T-cell epitopes from Cry j 1 and/or Cry j 2.

Stimulation with a peptide containing a T-cell epitope was not enough to induce detectable proliferation in PBMC of allergic patients in this study and previous reports [8, 10], while stimulation with Cry j 1 or rCry j 2 containing all T-cell epitopes induced proliferation (fig. 1). Concurrent presentation of several T-cell epitopes may be necessary to induce Th-cell activation in patient's PBMC, suggesting that possession of at least two or more types capable of present-

Table 4. Lymphokine secretion profiles of Cry j 1-specific TCCs which are classified into denoted groups by the types of restriction molecule[a]

Restriction	TCC	Epitope position	Lymphokine, pg/5×10^5 TCC/ml				Th type[b]
			IL-2	IFN-γ	IL-4	IL-5	
DRA1*01/DRB1*0901	PB11-21	151–165	4,190	20,000	4,510	16,300	Th0
	PB11-24	151–165	670	11,700	1,950	33,700	Th0
	PB6-37	151–165	<31	<31	49	61	Th?
	PB9-34	191–205	<31	86	1,680	13,900	Th2
DRA1*01/DRB5*0101	PB3-27	106–120	250	332	21,000	54,000	Th2
	PB8-2	106–120	190	2,110	5,710	6,800	Th0
	PB8-3	106–120	31	1,270	10,100	17,000	Th0
	PB9-39	106–120	48	51	5,120	25,400	Th2
	PB10-18	106–120	410	46	7,840	43,600	Th2
	PJ4-29	106–120	4,680	14,200	6,610	19,900	Th0
	PJ7-9	106–120	1,370	1,040	12,200	32,900	Th2
DQA1*0102/DQB1*0602	PB8-1	16–30	<31	<31	814	240	Th2
	PJ4-6	16–30	<31	1,500	334	4,210	Th0
	PJ1-27	91–105	32	1,220	224	788	Th0
	PJ5-6	146–160	1,500	1,170	5,920	20,300	Th0
	PJ5-9	146–160	1,720	825	266	14,400	Th0
	PB1-8	191–205	820	188	1,760	33,900	Th0
	PB8-4	251–265	44	36	4,050	29,900	Th2
	PJ4-20	326–340	560	3,080	<31	1,550	Th1
DPA1*01/DPB1*0501	PB9-37	61–75	<31	<31	7,760	23,800	Th2
	PB10-24	61–75	39	151	4,500	27,000	Th2
	PB2-14	211–225	<31	376	2,320	1,520	Th0
	PB4-20	211–225	<31	<31	133	487	Th?
	PB7-2	211–225	84	2,740	2,080	27,000	Th0
	PB8-32	211–225	<31	4,870	1,840	14,000	Th0
	PB8-34	211–225	78	14,800	3,040	24,100	Th0
	PB10-4	211–225	<31	<31	4,170	165	Th2
	PB11-23	211–225	<31	3,990	1,260	6,130	Th0
	PB11-26	211–225	32	1,100	6,520	19,000	Th0

[a] TCCs stimulated with no peptide in the presence of MMC-treated autologous EBV-B cells secreted undetectable level of those lymphokines.

[b] Definition of the Th-like cells: Th2 if the IL-4/IFN-γ secretion ratio was >10; Th1 if IFN-γ/IL-4 secretion ratio was >10; Th0 if there were approximately comparable IL-4 and IFN-γ secretion; and Th? if there were no detectable IL-4 and IFN-γ and if there was either detectable IL-4 (<310 pg/ml) and no detectable IFN-γ secretion or nodetectable IFN-γ and detectable IL-4 (<310 pg/ml) secretion.

ing T-cell epitopes in Cry j 1 and Cry j 2 such as DRA1*01/DRB5*0101, DRA1*01/DRB4*01, DQA1*0102/DQB1*0602, and DPA1*01/DPB1*0501 appear in the dictation toward the activation of individual Th cells reactive to each T-cell epitope. On the contrary, the gene products of DRA1*01/DRB1*0802, DRA1*01/DRB1*1501, DRA1*01/DRB4*01, DQA1*0401/DQB1*0402, DQA1*0301/DQB1*0303, and DPA1*01/DPB1*0201 could not present T-cell epitopes from Cry j 1 sequence and also DRA1*01/DRB1*0405, DQA1*0401/DQB1*0402, and DQA1*0301/DQB1*0303 could not present them from Cry j 2. This may, firstly, be due to existence of no antigenic peptides capable of binding to such molecules in Cry j 1 and Cry j 2 sequences. Secondly, antigen presentation by these molecules might be suppressed by antigen-specific CD8+ T cells [29, 30] or unknown gene regulations which participate in the antigen presentation by such types to Th cells [24]. It is apparent that HLA class II types capable of presenting T-cell epitopes in Cry j 1 and Cry j 2 sequences are associated with the Th-cell reactivity to the Japanese cedar pollinosis.

Table 5. Lymphokine secretion profiles of Cry j 2-specific TCCs which are classified into denoted groups by the types of restriction molecule[a]

Restriction	TCC	Epitope position	Lymphokine, pg/5 × 10^5 TCC/ml				Th type[b]
			IL-2	IFN-γ	IL-4	IL-5	
DRA1*01/DRB1*0901	PB5-29	16–30	<31	503	97	120	Th0
	PB11-40	16–30	<31	<31	50	3,940	Th?
	PB14-4	16–30	<31	<31	<31	46	Th?
	PR1-20	151–165	<31	<31	<31	2,280	Th?
	PR4-39	151–165	<31	<31	<31	1,150	Th?
	PR2-34	321–335	57	1,990	464	15,700	Th0
DRA1*01/DRB1*1501	PB12-33	36–50	<31	>8,000	<31	668	Th1
	PR2-25	36–50	47	<31	977	1,840	Th2
	PR5-40	36–50	1,150	1,330	355	14,900	Th0
	PB5-3	236–250	2,570	>8,000	525	33,000	Th1
DRA1*01/DRB4*01	PB14-34	186–200	186	420	93	77,500	Th0
	PC3-40	186–200	<31	<31	379	1,240	Th2
DRA1*01/DRB5*0101	PB3-32	66–80	<31	<31	323	1,630	Th2
	PB4-21	66–80	<31	109	239	13,300	Th0
	PB4-22	66–80	<31	483	158	6,760	Th0
	PC1-8	66–80	<31	2,710	32	903	Th1
	PC3-21	66–80	<31	<31	338	9,610	Th2
	PR4-20	66–80	<31	312	338	6,350	Th0
DQA1*0102/DQB1*0602	PR3-30	326–340	31	106	<31	57	Th?
	PR5-18	326–340	<31	<31	<31	64	Th?
	PB12-8	341–355	<31	3,210	<31	460	Th1
	PB14-19	341–355	<31	3,730	<31	882	Th1
	PR2-31	341–355	<31	<31	332	1,110	Th2
	PR5-12	341–355	<31	<31	2,528	3,100	Th2
	PB13-38	346–360	<31	2,020	<31	442	Th1
DPA1*01/DPB1*0201	PB14-5	181–195	87	126	469	1,210	Th0
	PB14-13	181–195	<31	59	2,440	37,800	Th2
DPA1*01/DPB1*0501	PB11-32	81–95	138	60	2,090	24,200	Th2
	PB13-18	81–95	<31	3,320	231	1,950	Th1
DPA1*0202/DPB1*0501	PC1-13	336–350	<31	<31	<31	2,130	Th?

[a, b] Refer to the legends in table 4.

It is generally accepted that Th2-like cells are preferentially induced from PBMC of allergic patients to the stimulation with certain allergens [11–14, 28]. However, why Th2-like cells are preferentially induced in PBMC of allergic donors is far from understood. In our observations, Th2- rather than Th1-like cells were preferentially induced under stimulation with Cry j 1, while an almost equal number of Th2- and Th1-like cells was induced by rCry j 2 stimulation (tables 4, 5). To elucidate this observation, we firstly analyzed whether peptide specificity and HLA class II restriction could dictate differentiation of Th cells into Th1- or Th2-like cells, since it has been reported that MHC genotype dictates the capacity of ligand density, resulting in the preferential induction of Th1 or Th2 cells in mice [31, 32], and various types of the gene products of HLA class II DR, DQ, and DP loci could present T-cell epitopes in Cry j 1 and rCry j 2 in this study and in Der p 1 [18], Der p 2 [19, 27], Fel d 1 [20] and so on. Our results indicated that induction of Th subsets in the TCCs specific to either Cry j 1 and Cry j 2 was neither associated with peptide specificity and HLA class II restriction or directly regulated by DR, DQ, and DP molecules derived from different HLA class II loci (tables 4, 5). Identification of T-cell epitopes in Lol p 1 [28] or Bet v 1 [14] utilizing TCCs generated from allergic patients and

nonallergic individuals suggested that there was no correlation between peptide epitope reactivity and lymphokine secretion pattern of the TCC, although HLA class II restrictions for each TCC were not determined. The same suggestion was proposed by a study of the comparison between Der p 2p20-33-specific TCCs restricted by DQB1*0602 generated from an allergic patient and a nonallergic individual [33]. Our observations using TCCs specific to Cry j 1 or rCry j 2 are essentially consistent with the previous suggestion [14, 28, 33]. Taken together, neither a combination of an antigenic peptide and one HLA class II restriction, nor restriction molecules themselves directly regulate differentiation of Th cells from allergic donors toward Th2-like cells.

Secondly, it was reported that Th cells from allergic donors produced high levels of IL-4 in stimulation with low concentration of allergens, while they produced low levels of IL-4 in stimulation with high concentration of them [34]. In mice, a very low dose of peptide induced dominant IL-4 production, whereas a high dose selectively primed an IFN-γ response [35]. Binding affinities of the peptides toward MHC class II molecule regulated the preferential induction of Th subsets; low affinity binding of the peptide to the MHC resulted in preferential induction of Th2 cells, while high affinity binding dictated toward induction of Th1 cells in mice [36–38]. Another difference found in this study was requirement of different doses of Cry j 1 and Cry j 2 to induce threshold proliferation in PBMC. The difference and distiguishable difference in induction of Th subsets between TCCs specific to Cry j 1 and Cry j 2 seem to be closely related together. Requirement of higher dose of Cry j 1 than Cry j 2 to induce threshold response in PBMC permits an interpretation that the binding affinities of each antigenic peptide in Cry j 1 toward particular HLA class II molecules may be lower than those in Cry j 2, resulting in prefential induction of Th2-like cells in response to Cry j 1 and induction of an equal number of Th1- and Th2-like cells in Cry j 2. To confirm this, measurement of binding affinities of individual antigenic peptides to the particular HLA class II types following determination of the subsets of Th cells after stimulation with those peptides will be necessary.

Finally, limited types of HLA class II molecules capable of presenting T-cell epitopes in Cry j 1 and Cry j 2 mainly function as one of genetic backgrounds for the recognition of Cry j 1 and Cry j 2. We suggest a possibility that these types indirectly participate, to some extent, in selective induction of the subset of Th cells in patient with cedar pollinosis.

A higher level of secretion of IL-5 than IL-2, IFN-γ, and IL-4 by TCCs specific to Cry j 1 as well as Cry j 2 was observed. The main function of IL-5 is defined as a maturation and growth factor for eosinophils and basophils and as a chemotactic factor for eosinophil infiltration from blood vessels to the nasal airway [39]. IL-5 also functions as an inducer of intracellular adhesion molecule-1 (ICAM-1) gene expression in human nasal mucosa during nasal allergic reactions [40]. A high level of IL-5 secretion by TCCs might participate as one of the factors in the immunopathology of Japanese cedar pollinosis.

Recently, it was demonstrated that the injection of a peptide containing T-cell epitopes induced peripheral tolerance and moderated the symptoms of allergic diseases in experimental murine models [41, 42] and in man in vivo [43]. Identification of T-cell epitopes in Cry j 1 and Cry j 2 sequences, determination of restriction molecules, and consideration of possible roles of the molecules in selective induction of subset of Th cells can be helpful for the design of efficacious peptide-based immunotherapeutics for the management of Japanese cedar pollinosis [10].

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